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CELL-MEDIATED IMMUNE RESPONSES OF GUINEA PIGS TO AN INACTIVATED--ETC(U)
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The ability of a killed phase I *Coxiella burnetii* vaccine to induce cell-mediated immune (CMI) responses in guinea pigs was studied. Cell-mediated immune responses were assessed by the inhibition of macrophage migration (IMM) and lymphocyte transformation (LT) assays. The IMM response occurred rapidly, was detected at high levels, but was relatively short-lived. In contrast, the LT response developed more slowly, and persisted for a longer period. The vaccine given in a single dose, or two doses one week apart, protected guinea pigs from a subsequent virulent challenge.

Cell-Mediated Immune Responses of Guinea Pigs to an Inactivated Phase I *Coxiella burnetii* Vaccine

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Running head: C. BURNETII VACCINE-INDUCED IMMUNITY

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

5 August 1977

ABSTRACT

The ability of a killed phase I Coxiella burnetii vaccine to induce cell-mediated immune (CMI) responses in guinea pigs was studied. Cell mediated immune responses were assessed by the inhibition of macrophage migration (IMM) and lymphocyte transformation (LT) assays. The IMM response occurred rapidly, was detected at high levels, but was relatively short-lived. In contrast, the LT response developed more slowly, and persisted for a longer period. The vaccine given in a single dose, or two doses one week apart, protected guinea pigs from a subsequent virulent challenge.

Vaccines made from killed Coxiella burnetii have been shown to be effective in eliciting humoral antibodies in man and guinea pigs (19). The protective efficacy of the vaccine has been demonstrated in man and guinea pigs (2, 13, 21). Early Q fever vaccines were prepared from strains of C. burnetii predominantly in phase II; however, studies by Ormsbee et al. (16) showed that formalin-killed phase I rickettsiae possessed protective potencies 100 to 300 times greater than phase II organisms. Recent studies indicate that cell-mediated immunity (CMI) also may play a role in the defense against C. burnetii infection. Peritoneal macrophages from guinea pigs vaccinated with formalin-killed phase I rickettsiae are capable of killing ingested phase I rickettsiae in vitro in the absence of immune serum (9, 10). This activity is demonstrable at a time when macrophage inhibition factor (MIF) is present in the peritoneal cavity (10). In guinea pigs infected with phase I C. burnetii inhibition of macrophage migration (IMM) was noted as early as 3 days post challenge and reached maximum levels between 14 and 21 days, and at a time when there is no detectable circulating antibody to phase I antigen (11). Recently, Jerrells et al. (8) demonstrated marked lymphocyte transformation (LT) responsiveness in humans exposed to C. burnetii as long as 8 years previously.

In an effort to gain further insight into the cellular immune responses of animals vaccinated with a killed vaccine, we studied the IMM and LT responses in guinea pigs following vaccination. Our objective was to determine whether either response would prove to be a predictive correlative of vaccine-induced immunity to Q fever infection.

MATERIALS AND METHODS

Rickettsiae. The third egg passage (EP-3) of the phase I Henzerling strain of C. burnetii and the 88th egg passage (EP-88) of a phase II Nine-Mile strain were grown in yolk sacs of embryonated eggs as previously described (17).

Guinea pigs. Outbred male Hartley strain guinea pigs, weighing approximately 350 to 450 g, were obtained from Buckberg Lab Animals, Tompkins Cove, N.Y. All animals were provided water and commercial guinea pig chow ad libitum.

Vaccination. The vaccine consisted of a purified formalin-inactivated, particulate, Henzerling strain phase I C. burnetii (NDBR 105, Lot 4) produced by the Merrell National Laboratories, Swiftwater, PA (20). Guinea pigs were vaccinated with 30 μg of antigen, a dose reported by Fiset (5) to evoke humoral antibody production and protect humans against an aerosol challenge with phase I rickettsiae 10 months later. Guinea pigs allocated into three groups were given 30 μg of the antigen subcutaneously (s.c.) by one of the following regimens: (i) one group was vaccinated once with 1.0 ml of vaccine; (ii) another group, with 0.5 ml on day 0 and again 7 days later; and (iii) the third group, with 1.0 ml of the vaccine mixed with an equal volume of incomplete Freund's adjuvant (IFA) (Difco Laboratories, Detroit, MI).

Serological assays. Blood was collected from animals at selected times and serum antibody activity against phase I and II C. burnetii was determined by the indirect immunofluorescent antibody technique of Bozeman and Elisberg (3).

Inhibition of macrophage migration. Peritoneal exudate cells

were collected 4 days after intraperitoneal inoculation of guinea pigs with 25 ml of sterile mineral oil (Marcol) no. 90, Humble Oil and Refining Co., Houston, TX). The peritoneal exudate cells were harvested and processed as previously described (11). The agarose technique for estimating direct IMM was performed as previously described (11). The area traversed by the migrating cells were measured and the following formula was used to express the IMM.

% inhibition of macrophage migration =

$$100 - \frac{\text{mean area of migration with antigen}}{\text{mean area of migration without antigen}} \times 100$$

Lymphocyte transformation assay. Lymphocyte transformation was measured using a whole blood technique (Kenyon, R. H., M. S. Ascher, R. A. Kishimoto, and C. E. Pedersen, Jr., submitted for publication). Weekly samples of cardiac blood from individual animals was collected in tubes containing 0.2 mg of heparin per milliliter of blood and diluted 1:10 in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with penicillin (200 units/ml) and streptomycin (200 μg/ml). Diluted blood (200 μl) was dispensed into U-well microtiter plates (Cooke Laboratory Products, Alexandria, VA). Six replicate wells were prepared containing 25 μl volumes of one of the following components: (i) RPMI medium; (ii) 2 x 10^7 formalin-killed phase I (EP-3 Henzerling strain) in RPMI medium; (iii) 4 x 10^6 phase II (EP-88 Nine-Mile strain) formalin-killed rickettsiae in RPMI medium; or (iv) phytohemagglutinin P (PHA) (Difco) diluted 1:100 in RPMI medium. After 4 days incubation at 37 C in a 5% CO_2 atmosphere, 0.02 μCi of [^{14}C]thymidine (50 mCi/ml, New England Nuclear, Boston, MA) was added to each well. Twenty-four hours

later, cells were harvested with a multiple automatic sampler harvester (Mash II, Microbiological Associates, Bethesda, MD). The cultures were aspirated with 30 ml water through a glass fiber filter paper and 50 ml absolute methanol. Dried filters were immersed in scintillation vials with 5 ml touluene-"Liquifluor". (New England Nuclear) for scintillation counting. Stimulation indices (SI) were calculated by the following formula:

$$\frac{\text{cpm of stimulated culture}}{\text{cpm of control culture}}$$

All cpm are given as the geometric mean of the 6 replicate cultures. Preliminary experiments indicated that the average standard deviation for each set of cultures was approximately 40% of the mean, and 2-fold differences were significant at the 5% level.

RESULTS

Following vaccination of guinea pigs with the formalin-killed, particulate phase I, Q fever vaccine, the temporal development of humoral antibody and CMI responses was studied. The time of appearance and persistence of humoral and cellular responses were dependent upon the vaccination regimen. The IMM responses of guinea pigs given vaccine in a single dose is shown in Fig. 1. Migration of macrophages was inhibited significantly (40-50%) by either phase I or phase II C. burnetii antigens at 7 and 14 days postvaccination; but was unaffected by antigen on day 21. In comparable cultures with cells from nonvaccinated control guinea pigs IMM did not exceed 5%. In contrast to the early cellular immune response, serum antibody to phase II antigen did not appear until day 14 and antibody to phase I antigen was not detected by day 21 (Fig. 1).

Guinea pigs responded to the two-dose vaccine regimen with enhanced cellular and humoral responses compared to those given a single dose. Seven days after administration of the second dose, IMM was approximately 70% in cultures with either antigen (Fig. 2). Inhibition decreased thereafter, but remained demonstrable for 28 days compared to a 14 day duration after the single dose schedule. Likewise, production of antibodies was also potentiated with the two-dose regimen. Phase II antibody was detected after 7 days, and phase I antibody was detected on day 21 (Fig. 2).

The humoral and cellular responses were also determined in guinea pigs vaccinated once with the vaccine emulsifed in an equal volume of IFA. These animals demonstrated IMM and humoral antibody responses similar to animals given two-doses of the vaccine a week apart (data not shown).

Lymphocyte transformation. Antigen-specific responsiveness of peripheral leukocytes from vaccinated guinea pigs was evaluated with the LT test. These responses were measured on the same animal at weekly intervals. Animals inoculated with a single dose of vaccine developed a relatively low, delayed LT response; the SI did not differ from baseline on day 7 or 14, but increased significantly on day 21 and persisted at an elevated level until day 35 (Fig. 3). In contrast, with the group vaccinated on the two-dose schedule, rickettsial antigens failed to stimulate a proliferative response at any time during the 42-day experimental period (data not shown).

Additional studies were performed with a group of animals immunized with vaccine in IFA. These guinea pigs demonstrated markedly enhanced leukocyte responsiveness. The response was

minimal at 7 days (Fig. 3), but increased abruptly in the presence of either phase I or II antigen on day 14, and persisted at relatively high levels until day 42, when the experiment was terminated. In contrast, the SI in cultures from animals given IFA without antigen remained at baseline values throughout the experimental period. Stimulation indices for PHA control cultures ranged between 20 and 40 at each sampling time.

Responses of vaccinated animals to a virulent challenge. The efficacy of the vaccine given either in a single dose or two doses a week apart was determined by in vivo protection studies. Evaluation of a protective effect was based upon inhibition of a febrile response (> 40 C). Animals were challenged intraperitoneally (i.p.) 21 days after vaccination which approximately 10^5 EID_{50} of live phase I *C. burnetii*. Rectal temperatures of all guinea pigs vaccinated with either one or two doses, or one dose of the vaccine in IFA remained within normal range after challenge, whereas all non-vaccinated animals became febrile by days 3-5 post challenge (Table 1). Expanded studies on protection are being performed by one of us (JWJ). Humoral and cellular immune responses of vaccinated animals were also examined at selected times following challenge with virulent rickettsiae. Earlier results indicated that guinea pigs vaccinated with one dose of the vaccine had no demonstrable serum antibody to phase I antigen and there was no IMM response with either phase I or phase II antigen (Fig. 4). As early as one day after challenge, IMM became evident and reached significantly higher levels 3 days later; this response diminished by day 7 and was no longer present on day 14. In contrast there was no phase I antibody present one day

later, but antibody titers for both phase I and II antigens were elevated on day 3, continuing to increase on day 7 and remained at high levels on day 14.

The LT responses were also determined for a comparable group of guinea pigs following challenge at 21 days. At the time of challenge (21 days) the SI value was approximately 5.0 (Fig. 3). Unlike the significant increase in the IMM responses, there was no significant elevation in the SI up to 14 days following challenge (data not shown).

DISCUSSION

Guinea pigs vaccinated s.c. with formalin-killed, particulate, phase I _C_. _burnetii_ developed a CMI response as measured by two different in vitro assays. The IMM activity was demonstrable at very high levels within one week after vaccination but was relatively short-lived. This response to s.c. vaccination is similar to that observed in guinea pigs after infection with virulent phase I _C_. _burnetii_ by the respiratory route (11).

The temporal course of the antigen-specific response as measured by the LT test did not correlate with IMM. After one-dose of vaccine LT response developed more slowly than IMM, but persisted at low levels for a longer period; immunocompetent populations of lymphocytes were present in peripheral circulation 35 days after vaccination. After two doses of vaccine, the blastogenic response failed to be detectable, although IMM reactivity was markedly enhanced. This lack of response might be explained by the hypothesis put forth by Oppenheim (15) to antigen-antibody complexes formed in

antibody excess, and further elaborated by Lee and Sigel (12) that antigens complexed with IgM antibodies can depress lymphocyte stimulation. The observation that antibody to phase I antigen was present in guinea pigs that received two doses of vaccine, but not in those that received one dose would seem to be in accordance with this hypothesis. However, although antibody titers for guinea pigs that were inoculated with one dose of the vaccine suspended in IFA were similar to those for the two-dose group, the blastogenic response after vaccine in Freund's adjuvant was remarkably enhanced. This suggests that the lack of SI response for the two-dose regimen was not due wholly to antibody suppression.

The ability of killed rickettsial vaccines to induce a cellular immune response has been noted by others. Coonrod and Shepard (4) reported that lymphocytes from humans vaccinated or infected with spotted fever or typhus group rickettsiae exhibited in vitro LT when cultured with specific antigen. Kenyon et al. (submitted for publication) found that formalin-killed Rickettsia rickettsii produced negligible LT but high levels of IMM in guniea pigs.

The present studies contribute additional evidence of a role for CMI in protection; guinea pigs vaccinated with one dose of the antigen produced no serum antibody against phase I Coxiella, developed antigen-specific lymphocyte responsiveness and were protected against challenge 21 days after vaccination. Suggestive evidence for a role for CMI in resistance to infection with C. burnetii was described in our previous studies (9, 10) in which phase I formalin-killed antigen were capable of destroying phase I rickettsiae in the absence of

immune serum. Likewise, Benenson (2) showed that whereas only 50% of human volunteers vaccinated with killed phase I antigen developed detectable antibody, all were resistant to aerosol challenge with virulent rickettsiae.

The role of lymphokines, such as MIF and blastogenic factor, in the pathogensis of and/or, host defense against Q fever infections is not known. Enhancement of antigen-specific blastogenic responses could result in an increased production of functional T and B lymphocytes and/or in stimulated production of lymphokines or antibodies capable of affecting host resistance in a variety of ways. It has been suggested by other investigators that MIF not only localizes macrophages at the site of infection, but also participates in the activation of macrophage; phagocytic, bacteriostatic, and even bactericidal activities of macrophages are enhanced in the presence of either sensitized lymphocytes or their soluble products (6, 14, 18). Studies by Hinrichs and Jerrells (7) provide evidence that normal guinea pig peritoneal macrophages cultured in vitro with immune lymphocytes or with MIF-rich supernatant fluid inhibit the growth of ingested C. burnetii.

Our data suggesting a role for CMI does not eliminate a contributory role for humoral antibody in the overall defense of the host. Serum antibodies for phase II antigen were present at high levels in those guinea pigs given two doses of the vaccine and antibodies against phase I antigen rapidly increased in vaccinated animals following challenge. In our previous studies pretreatment of rickettsiae with immune serum not only enhanced ingestion of organisms within the phagocyte but also potentiated the destruction of ingested organisms (9, 10). Moreover, while immune serum is not

known to have a direct rickettsicidal effect on C. burnetii, rickettsiae incubated with specific antiserum prior to inoculation do not cause infection in guinea pigs (1). The rapid mobilization of both humoral and cellular responses after challenge of vaccinated guinea pigs suggests that both facets of immune activity participate in defense against Q fever infection. Further work is needed to define the specific contribution of humoral and cellular immune responses in protection.

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FIGURE LEGENDS

FIG. 1. Percent migration-inhibition and humoral antibody responses of guinea pigs following one dose of *C. burnetii* vaccine. Mean and standard error of the mean of 4 to 6 animals.

FIG. 2. Percent migration-inhibition and humoral responses of guinea pigs following two doses of *C. burnetii* vaccine. Mean and standard error of the mean of 4 to 6 animals.

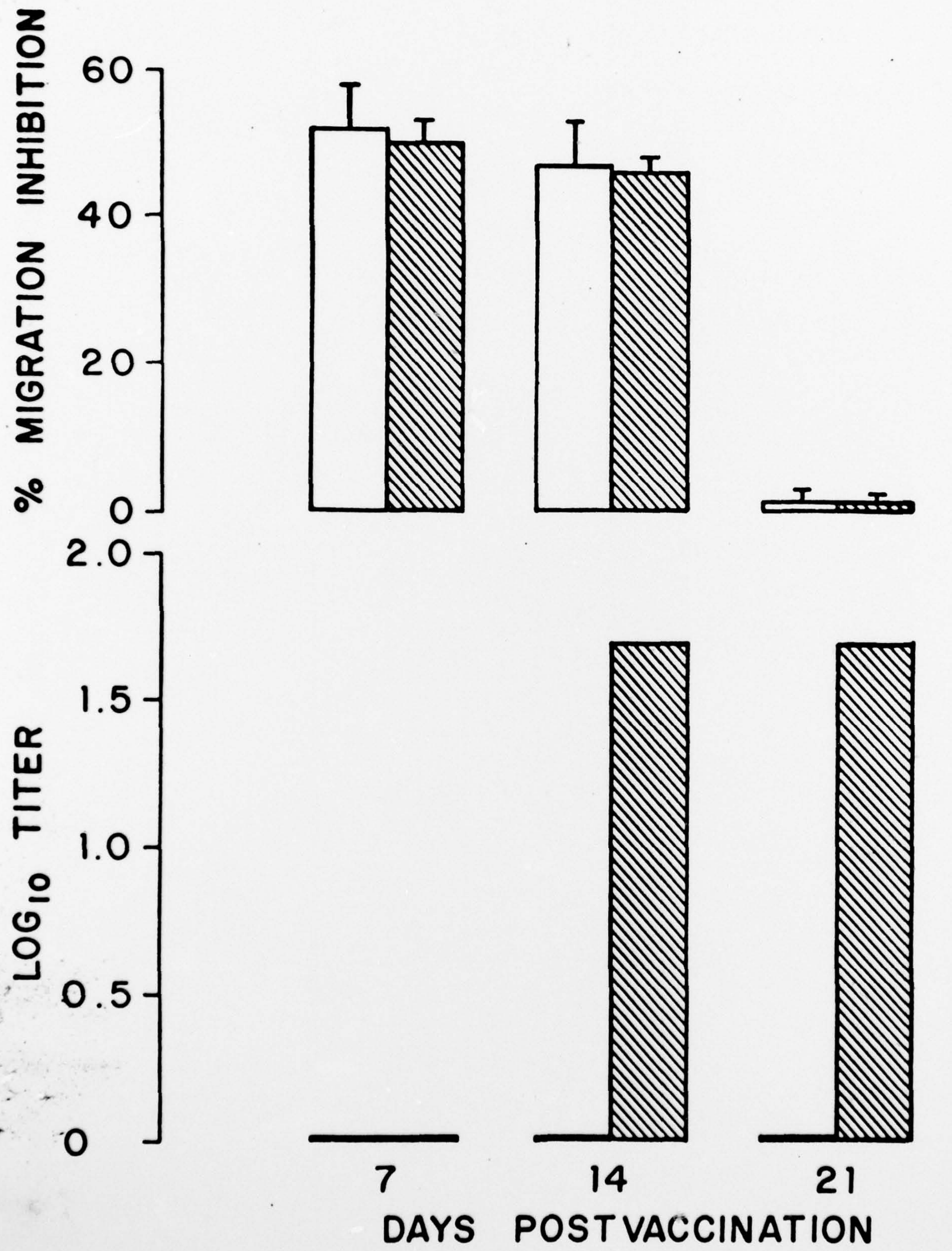
FIG. 3. Stimulation indices of guinea pigs following vaccination with 1 dose of *C. burnetii* vaccine with and without incomplete Freund's adjuvant. Mean of 4 to 12 animals.

FIG. 4. Percent migration-inhibition and humoral and responses of vaccinated guinea pigs (1 dose) following challenge with phase I *C. burnetii*. Mean and standard error of the mean of 4 to 8 animals.

TABLE 1. Protection of vaccinated guinea pigs against an i.p. challenge of phase I *C. burnetii*

Treatment	Febrile Response[a]
Single dose of vaccine	0/10
Two dose a week apart	0/10
Single dose of vaccine emulsified in IFA	0/10
Nonvaccinated	10/10

[a]Protection based on absence of a febrile response (>40°C).



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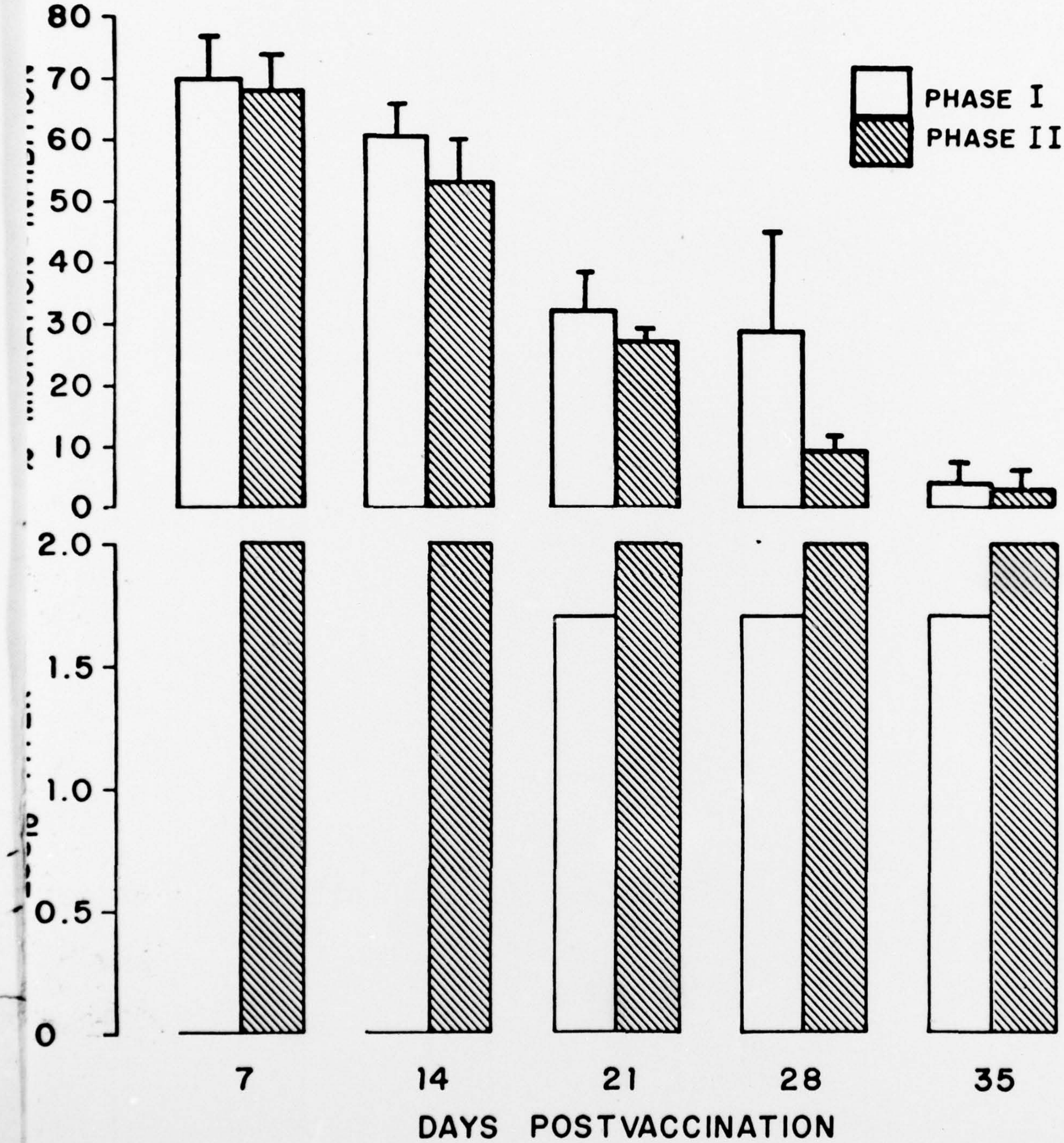
DAYS POSTVACCINATION



PHASE I
PHASE II
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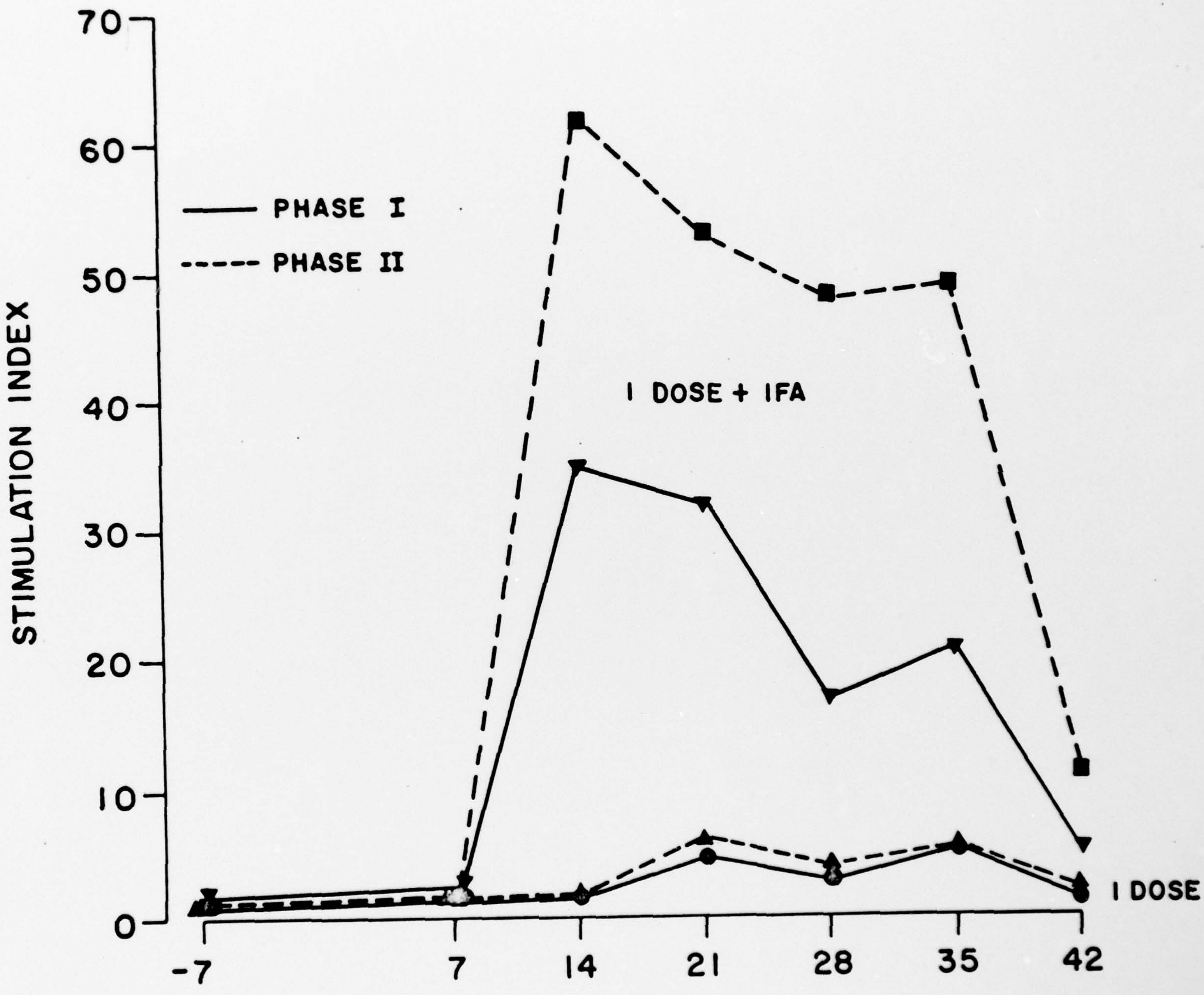
DAYS POSTVACCINATION



STIMULATION INDEX
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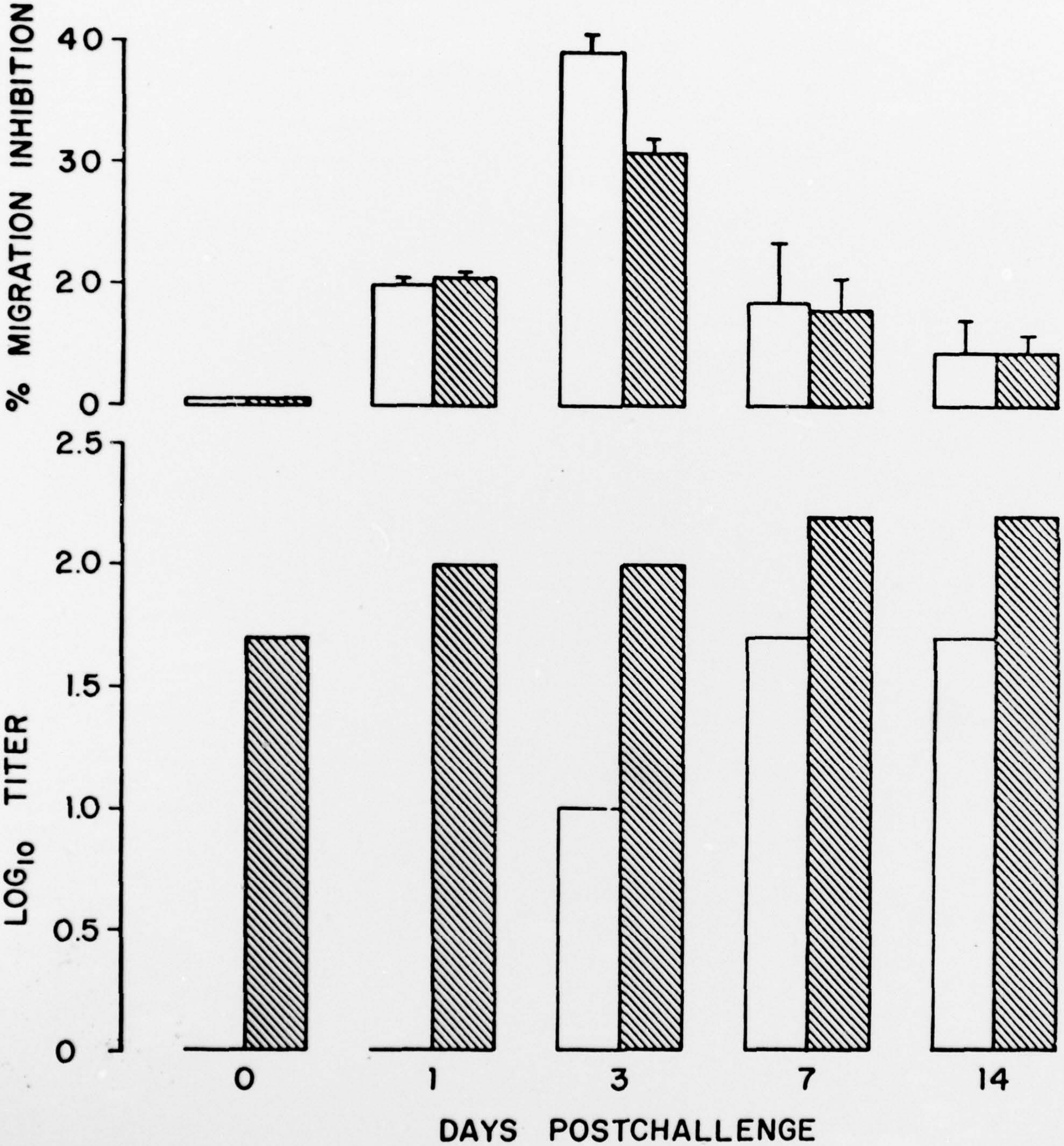
DAYS POSTVACCINATION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